REMARKS

Applicants request entry of the above amendments pursuant to the provisions of 37 C.F.R. §1.116(b), reconsideration of the present application in view of the foregoing amendments, and allowance of all of the remaining claims.

Claim Amendments

Claims 1-4, 10-15, 18-23, 26-31, 34-39, and 42-47 currently are pending.

Claims 5-9, 16-17, 24-25, 32-33, 40-41, and 48-55 were previously cancelled.

Claims 42-47 are cancelled in this amendment without prejudice. Claim 42 depends on Claim 5 which was previously cancelled. Claims 44 and 46 depend on Claim 6 which was also previously cancelled.

Claims 10-11, 15, 18-19, 23, 26-27, 31, 34-35, and 39 stand withdrawn as being drawn to non-elected invention.

Applicants request amendments to Claims 1-4 to replace the term "eradicate" with "treat." No new matter is added by this amendment. Support for this amendment can be found throughout the application as filed. For example, support for the amendment can be found on page 7, lines 34-35 of the application as filed.

Insofar as the above amendments place the application in better form for appeal, entry of these amendments under the provisions of 37 C.F.R. §1.116(b) is proper. Entry of these amendments is requested.

The cancellation and/or amendment of the subject matter is not intended to be a dedication of the subject matter to the public. Applicants reserve the right to file one or more continuation, divisional, or continuation-in-part application to the cancelled subject matter.

After amending the claims as set forth above, Claims 1-4, 12-14, 20-22, 28-30, and 36-38 are under consideration.

This amendment adds, changes and/or deletes claims in this application. A detailed listing of all claims that are, or were, in the application, irrespective of whether the claim(s) remain under examination in the application, is presented, with an appropriate defined status identifier.

Note

Applicants thank the Office for pointing out that no rejection was made under 35 U.S.C. §112, first paragraph-enablement, in the last Office Action dated 10/09/2007. Applicants also thank the Office for withdrawing the rejection of claims 3, 4, 28-30, 36-38, and 44-46 under 35 U.S.C. §112, second paragraph.

Claim rejection under 35 U.S.C. §103(a)

Claims 1-4, 12-14, 20-22, 28-30, 36-38, and 44-46 stand rejected under 35 U.S.C. §103(a) as being unpatentable over Futami *et al.* (*J. of Immunotherapy*, vol. 12, 247-255, 1992) in view of Wilson *et al.* (*Int. J. Radiation Oncology Biol. Phys.*, vol. 42, 905-908, 1998) and in view of Olsson *et al.* (*Int. Immunology*, vol. 10, 499-506, 1998). The Office alleges that Futami *et al.* teach a method of treating tumor by 5-methyl XAA in conjunction with a T-cell stimulating molecule, IL-2; Wilson *et al.* teach DMXAA which potentiates tumor radiation response compared to each treatment alone; and Olsson *et al.* teach that human IL-2 is induced by CD80 (B7.1, a CAM molecule) in cancer cells and T cells. The Office further alleges that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the claimed invention was made to replace the IL-2 with its stimulator (CAM) and an analogue of XAA with DMXAA. *See* pages 3-4 of the Office Action.

Applicants traverse the rejection for the following reasons:

The Supreme Court in *KSR International Co. v. Teleflex Inc.*, 127 S.Ct. 1727 (2007) reviewed the analysis for determining if an invention is obvious over the teachings of the prior art and affirmed the factual analysis set forth in *Graham v. John Deere Co. of Kansas City*, 383 U.S. 1, 17-18 (1966). *KSR*, 127 S. Ct. at 1734 (quoting *Graham*, 383 U.S. at 17-18). The factual inquiries necessary in an analysis of obviousness by the Office is delineated in MPEP § 2141 as follows:

> (A) Determining the scope and contents of the prior art;
>
> (B) Ascertaining the differences between the prior art and the claims in issue;
>
> (C) Resolving the level of ordinary skill in the pertinent art; and
>
> (D) Evaluating evidence of secondary considerations...

MPEP § 2141 further states that:

> When applying 35 U.S.C. 103…
>
> (A) The claimed invention must be considered as a whole;
>
> (B) The references must be considered as a whole and must suggest the desirability and thus the obviousness of making the combination;
>
> (C) The references must be viewed without the benefit of impermissible hindsight vision afforded by the claimed invention; and
>
> (D) Reasonable expectation of success is the standard with which obviousness is determined.

Application of the obviousness analysis of KSR, supra., was discussed in the recent decision by the Court of Appeals for the Federal Circuit in *Takeda Chemical Industries Ltd. v. Alphapharm Pty. Ltd.*, 83 USPQ2d 1169 (CAFC 2007). The CAFC held that:

> "[w]hile the KSR Court rejected a rigid application of the teaching, suggestion, or motivation ("TSM") test in an obviousness inquiry, the Court acknowledged the importance of identifying "a reason that would have prompted a person skilled in the relevant field to combine the elements in the way the claimed new invention does" in an obviousness determination. KSR, 127 S. Ct. at 1731. Moreover, the Court indicated that there is "no necessary

> inconsistency between the idea underlying the TSM test and the *Graham* analysis". *Id.* As long as the test is not applied as a "rigid and mandatory" formula, that test can provide "helpful insight" to an obviousness inquiry. *Id.*"

After application of the facts of Applicants' invention in the context of the Graham analysis and consistent with the ruling in *KSR*, it will be shown that Applicants' claimed invention meets the criteria of 35 U.S.C. § 103.

Some of the salient features of the Applicants' invention include treatment of large or advanced tumors[1], administration of B7.1 with DMXAA, and synergy between B7.1 and DMXAA since neither of B7.1 or DMXAA are effective when used alone. In addition, as per independent Claim 4 and dependent Claims 12, 13, 20, 21, 28, 29, 36, and 37, a further salient feature includes administration of B7.1 prior to the administration of DMXAA.

(A) The Scope and Content of the Prior Art

Futami *et al.* disclose treatment of cancer with derivatives of xanthenone-4-acetic acid (XAA) and recombinant human interleukin-2 (rhIL-2). Futami *et al.* clearly state that of the three analogues of XAA tested, namely, 5-methyl-XAA, 5-chloro-XAA and 7-methyl-XAA, the 7-methyl-XAA showed no anti-tumor activity and showed no synergy with rhIL-2. *See* summary on p. 247 of Futami *et al.* Futami *et al.* do not teach treatment of large or advanced tumors. Futami *et al.* do not teach using DMXAA. Futami *et al.* do not teach using B7.1. Futami *et al.* do not teach administration of B7.1 prior to the administration of DMXAA to the patient.

Olsson *et al.* disclose a comparison of the dependency of CD80 versus CD86 in the induction of transcription factors regulating the human IL-2 promoter. Olsson *et al.* teach T-cell activation by CD80 and CD86 producing a host of heterogenous effects including T-cell proliferation, multiple cytokines, and multiple other proteins such as AP-1, NF-kB, CD28RE and NF-AT (*see* Olsson *et al.*, p. 504, left column). Olsson *et al.* do not teach treatment of cancer or

[1] "advanced or large tumors" defined as >0.5 cm on p. 16, lines 12-14 of the application as filed.

in particular, treatment of large or advanced tumors, using CD80. Olsson *et al.* do not teach combination therapy of large or advanced tumors using CD80 with DMXAA.

While admittedly, Wilson *et al.* teach enhancement of tumor radiation response by DMXAA, there is simply no teaching in Wilson *et al.* to combine DMXAA with CAM. And for the reasons noted in detail below, nor is there any basis in either Wilson *et al.* or the other cited references to combine their teachings in the manner necessary to arrive at the claimed invention.

(B) The Differences Between The Cited Art and The Claimed Invention

Applicants' claimed invention is directed to a method of treatment for a mammal, with advanced or large tumor burdens, comprising the administration to the mammal of a T-cell co-stimulatory cell adhesion molecule (CAM) in conjunction with a tumor growth restricting agent, either of which alone would be ineffective in treating an advanced or large tumor burden, wherein said CAM is B7.1 and wherein said tumor growth restricting agent is DMXAA.[2] Applicants' invention is based on the surprising results that the administration of these agents in combination is effective to treat advanced or large tumours and to generate anti-tumour systemic immunity.

Futami *et al.* teach analogues of XAA in combination with rhIL-2. Futami *et al.* do not teach treatment of large or advanced tumors of the claimed invention. Futami *et al.* do not teach DMXAA. Futami *et al.* do not teach administration of B7.1 in combination with DMXAA.

Futami *et al.* provide no suggestion or motivation to use just any analogue of XAA let alone DMXAA. In fact, Futami *et al.* teach away from using just any analogue of XAA. Futami *et al.* clearly state that of the three analogues of XAA tested, namely, 5-methyl-XAA, 5-chloro-XAA and 7-methyl-XAA, the 7-methyl-XAA showed no antitumor activity and showed no synergy with rhIL-2 (*see* summary on p. 247 of Futami *et al.*).

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[2] This brief summary is provided for illustrative purposes only and is not to be construed as limiting, modifying or altering the scope of each of the independent claims provided. The Examiner is requested to review the independent claims to determine the exact scope of the claimed invention.

> A prior art reference must be considered in its entirety, i.e., as a whole, including portions that would lead away from the claimed invention. *W.L. Gore & Associates, Inc. v. Garlock, Inc.*, 721 F.2d 1540, 220 USPQ 303 (Fed. Cir. 1983), *cert. denied*, 469 U.S. 851 (1984).

Therefore, Futami *et al.* considered as a whole clearly teach away from using just any analogue of XAA. A person of ordinary skill in the art can have no reasonable expectation of success to replace XAA analog of Futami *et al.* with DMXAA, more so since Futami *et al.* teach away from using just any analog of XAA. The fact that the analogue of XAA of Futami *et al.* cannot be just any analogue of XAA is further corroborated by the Office's own assertion[3] that finding synergistic combinations of drugs require extensive testing, i.e., is an art with no reasonable expectation of success (emphasis added):

> One skilled in the art recognizes that the search for combinations of drugs (each has less effect when it is used alone) exerting a combined effect requires a great deal of empirical testing if agents known to have anti-cancer properties or that may augment an agent having anti-cancer properties (Gerson *et al.*, WO 03/070234, page 2, lines 11-14). In addition, not all the analogue of XAA has a tumor restricted function, Futami *et al.*, (J of Immunotherapy, vol. 12, 247-255) indicates that 7-methyl XAA, a analogue of XAA, self, or combination with IL-2 has not synergistic activity in suppression of tumor growth (page 252-253, col. 1). Thus, it would be undue experimentation to test two agents in combination in order to determine whether one skilled in the art could use them together for treating a large or advanced tumor.

Therefore, the Office clearly acknowledges that search for combination of drugs requires great deal of empirical testing and that not all the analogues of XAA have a tumor restricted function. The Office states[4] that the above statement made in the enablement rejection was made for the claims reciting a method of treating advanced or large tumor with any analogue of XAA in combination with any CAM. Although Applicants agree that the above statement was made in the enablement rejection, the fact that, not all the analogues of XAA have a tumor restricted function, is a stand alone statement and holds true for the obviousness rejection too. Futami *et al.* clearly show that the 7-methyl XAA is not a successful tumor restricting agent, corroborating the statement that not all of the analogues of XAA can be effective. Therefore, a person of

[3] Office Action dated 08/23/06, p. 4

[4] Current Office Action dated 04/28/2008, p. 7, ¶ 2

ordinary skill in the art will not have any reasonable expectation of success in substituting an analogue of XAA of Futami *et al.* with DMXAA of instant claims.

Futami *et al.* do not teach large or advanced tumors of the claimed invention. Futami *et al.* disclose injecting tumor cells intrarenally and treating the injected animals only for 7 days after tumor injection (*see* Futami *et al.*, p. 249, ¶ 1). This is in contrast to the instant invention where tumor cells are injected into the left flank of the mouse followed by a long period of tumor growth of anywhere from 14 days to about 21 days resulting in large or advanced tumors (>0.5 cm). *See* application as filed, p. 13, paragraph tiled "Experimental Tumor Model." Given the short length of time for tumor growth in Futami *et al.* as well as the likely diffusion of tumor cells relative to the non-limiting model system of the instant invention, it is clear that Futami *et al.* do not teach treatment of large or advanced tumors.

The Office states[5] that, "... one of ordinary skill in the art would have been motivated with a reasonable expectation of success to optimize the treatment method according to the growth rate and condition of different tumors established in the mice comprising the tumor size and day or dose of the administration in order to get better result and response ...."

Applicants assert that the size of the tumor and the regimen of treatment is not simply a matter of optimization. Applicants point the Office to the background section of the application as filed, p.1, lines 10-28:

> Advanced cancers and large tumours burdens are refractory to treatment with therapeutic agents. Although these same agent may be effective against smaller tumours, their use does not achieve complete eradication of large tumour burdens. Large tumours can continue to grow unchecked, or their re-growth is not recongnised by the body's immune system.
>
> In addition, tumours acquire defensive and survival functions which limit the efficacy of therapeutic agents and/or the body's own immune response. For unknown reasons large tumor burdens appear to either impair or retard the generation of anti-tumour cytotoxic T lymphocyte responses. In immunotherapy, gene transfer of T cell co-stimulatory cell

[5] Office Action dated 01/16/2007, p. 6, end of ¶ 2

> adhesion molecules is effective against only very small tumours and only weak anti-tumours systemic immunity is generated.
>
> It is an object of the present invention to provide a therapeutic combination that will at least partially overcome the resistance of large tumour burdens to immunotherapy, or at least provide the public with a useful choice in the treatment of cancer.

It is known that the large tumors have been resistant to immunotherapy and that the agents that are effective for small tumors have not been effective for large tumors. Applicants provide Kanwar *et al.* (*Gene Therapy* **6**:1835-1844 (1999)) as **Exhibit I** that clearly shows that the immunotherapy that was effective for small tumors was found to be ineffective for large tumors. Kanwar *et al.* on p.1839, right hand column, last paragraph to p.1840, left hand column, first paragraph, states that:

> We have consistently found that gene transfer of CILs into tumors less than 0.3 cm in diameter causes rapid tumor rejection, whereas tumors become increasingly refractory to treatment as their size increases. Larger tumors appear actively to suppress or avoid the immune response, since challenge of cured animals with large tumor burdens led to reductions in the levels of antitumor CTL activity compared with the response to smaller burdens.

Kanwar *et al.* further show that the challenge with the large tumor burden of $5x10^7$ cells significantly suppressed the generation of anti-tumor CTL activity by as much as 46 to 80% compared with CTL activity generated with the small tumor burden ($1x10^5$ cells) (*see* Kanwar *et al.* p. 1836, right hand column).

Therefore, it is not a matter of simple optimization to use the agents that are effective for small tumors to use them against large tumors. In absence of any suggestion or motivation in Futami *et al.* to treat large or advanced tumors, a person of ordinary skilled in the art will not be motivated to treat large tumors with analogues of XAA since large tumors have been resistant to the immunotherapies that are effective against small tumors. Further, there is no reasonable expectation of success to a person of ordinary skill in the art to use an analogue of XAA for the treatment of large tumors.

Futami *et al.* do not teach administration of IL-2 prior to analogue of XAA. Instant independent Claim 4 and dependent claims 12, 13, 20, 21, 28, 29, 36, and 37 are directed to administration of CAM prior to (e.g., 12 to 48 hours) the administration of the tumor growth restricting agent. The instant specification shows[6] the surprising result that combined therapy by timed delivery of B7.1 and DMXAA eradicates large tumors:

> ... established tumours (0.6-0.8 cm in diameter) were first treated with B7.1 to stimulate anti-tumour immunity, and DMXAA and FAA were administered one day later to retard tumour growth. Remarkably, tumours rapidly diminished in response to the combination of B7.1 and DMXAA accompanied by massive necrosis, such that by the third week of treatment tumours had completely disappeared (FIG. 1b).

In contrast, Futami *et al.* teach the administration of tumor growth restricting agent one day before administration of rhIL-2 (*see* Futami *et al.*, p.249, ¶ 1). The Office states[7] that, it would be *prima facie* obvious to administer one reagent prior to another. Applicants contend that the Office has made an unsupported statement and request the Office to show evidence to support the statement. Contrarily, the evidence in Applicants' specification demonstrates that prior administration of B7.1 stimulates anti-tumour immunity which by subsequent administration of DMXAA/FAA retards tumor growth.

Nevertheless, there is no such teaching in Futami *et al.* of two step administration as per Claim 4 and the dependent claims noted above. In the absence of any teaching, suggestion or motivation in Futami *et al.*, a person of ordinary skill in the art will not be motivated to administer IL-2 before the administration of tumor growth restricting agent. There is no reasonable expectation of success to substitute the XAA analogue of Futami *et al.* with DMXAA and rhIL-2 with B7.1 as well as to substitute the treatment regimen of Futami *et al.* in order to arrive at the claimed invention.

Therefore, Futami *et al.* do not suggest or motivate a person of ordinary skill in the art with any reasonable expectation of success to substitute all the three features of Futami *et al.*,

[6] p. 16, lines 25-34 to p. 17, lines 1-7 of the application as filed.

[7] Office Action dated 08/23/2006, p. 8, end of ¶ 2

namely, small tumors with large or advanced tumors; analogues of XAA with DMXAA; and IL-2 with B7.1 to arrive at the claimed invention.

The Office alleges that Olsson *et al.* disclose an increase in IL-2 levels as a result of administration of CAM B7.1. Olsson *et al.* do not teach treatment of cancer. At best, Olsson *et al.* use cancer cell lines to describe the effect of two different CAMs, CAM B7.1 and CAM B7.2. Nowhere do Olsson *et al.* teach treatment of cancer using CAM B7.1. Further, Olsson *et al.* do not suggest or motivate a person of ordinary skill in the art to combine B7.1 with DMXAA for the treatment of cancer. Furthermore, Olsson *et al.* disclose T-cell activation producing multiplicity of heterogenous effects including T-cell proliferation, production of multiple cytokines (*see* Olsson *et al.*, "introduction"), and production of various other proteins such as multiple transcription factors including AP-1, NF-kB, CD28RE, and NF-AT (*see* Olsson *et al.* p. 504, left column). The administration of B7.1 of Olsson *et al.* would result in multiple effects, as described *supra*, of unknown magnitude (e.g., it is not clear how much of endogenous IL-2 would accumulate after the administration of B7.1) and unknown duration (e.g., it would be unclear as to how long the effect would last). This is in contrast to Futami *et al.* which discloses single exogenously administered purified cytokine IL-2 which would have known half life and other pharmacokinetics. Therefore, there is no reasonable expectation of success for a person of ordinary skill in the art to substitute an exogenously administered purified cytokine IL-2 of Futami *et al.* with B7.1 of Olsson *et al.* which endogenously produces a plethora of heterogenous effects one of which is IL-2 induction. Additionally, Olsson *et al.* do not suggest or motivate a person of ordinary skill in the art to use B7.1 in combination with DMXAA for the treatment of cancer.

Wilson *et al.* fail to fulfill the gap left out by Futami *et al.* and Olsson *et al.* Wilson *et al.* only disclose DMXAA as an anti-cancer agent. Wilson *et al.* do not teach combination of DMXAA with B7.1. There is no suggestion or motivation in Wilson *et al.* to combine DMXAA with B7.1.

The Office alleges that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the claimed invention was made to replace the IL-2 with its stimulator (CAM) and an analogue of XAA with DMXAA. Applicants submit that in the absence of any teaching, suggestion, or motivation in either of the cited references, a person of skill in the art will not be motivated to combine the reference teachings and come up with the claimed invention.

> A statement that modifications of the prior art to meet the claimed invention would have been "'well within the ordinary skill of the art at the time the claimed invention was made'" because the references relied upon teach that all aspects of the claimed invention were individually known in the art is not sufficient to establish a *prima facie* case of obviousness without some objective reason to combine the teachings of the references. *Ex parte Levengood*, 28 USPQ2d 1300 (Bd. Pat. App. & Inter. 1993). **">[R]ejections on obviousness cannot be sustained by mere conclusory statements; instead, there must be some articulated reasoning with some rational underpinning to support the legal conclusion of obviousness.'" *KSR*, 550 U.S. at ___, 82 USPQ2d at 1396 quoting *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1336 (Fed. Cir. 2006).<
>
> (Emphasis in original).

While the cited references may together teach some of the limitations of the claimed invention, as explained *supra*, there is no motivation to select these components in the particular combination to arrive at Applicants' claimed invention. Applicants submit that the Office has resorted to impermissible hindsight as the Office has cited three different arts to put together the elements of the claimed invention where none of the cited art individually or in combination teach, suggest or motivate a person of ordinary skill in the art with any reasonable expectation of success to arrive at the claimed invention.

(C) Evaluating evidence of secondary considerations

Assuming *arguendo*, that the Office has established a *prima facie* case of obviousness, the instant application shows surprising and unexpected results in treatment of large or advanced tumors by combining methods of immunotherapy with methods of chemotherapy previously demonstrated to be ineffective in the long term treatment of advanced or heavy tumour burdens. Such results rebut any such *prima facie* case. Specifically, the application as filed on p. 9, lines 9-22 discloses that:

> The anticancer agents flavone acetic acid (FAA) and 5,6-dimethylxanthenone-4-acetic acid (DMXAA) cause initial reductions in tumour size when administered, but tumours subsequently grow unchecked, and both reagents generate a weak and ineffective anti-tumour CTL response. DMXAA and FAA appear to exert their anti-tumour activities via several pathways including reduction of tumour blood flow leading to hemorrhagic necrosis and the induction of multiple immunomodulatory factors including cytokines, nitric oxide, and activated natural killer cells. However, neither agent is able to generate the desired anti-tumour systemic immunity, and they are ineffective against large tumour burdens.
>
> The finding made by the applicants that administration of these agents in combination is effective to both eradicated advanced or large tumours and to generate anti-tumour systemic immunity is therefore surprising and representative of a significant advance in cancer treatment.

Moreover, the evidence found in the application as recited above, demonstrates synergy which was neither taught nor suggested by the cited art. In light of the arguments as presented above, Applicants request the Office to withdraw this rejection based on 35 U.S.C. §103(a).

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

CONCLUSION

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

The Commissioner is hereby authorized to charge any additional fees which may be required regarding this application under 37 C.F.R. §§ 1.16-1.17, or credit any overpayment, to Deposit Account No. 19-0741. Should no proper payment be enclosed herewith, as by a check or credit card payment form being in the wrong amount, unsigned, post-dated, otherwise improper or informal or even entirely missing, the Commissioner is authorized to charge the unpaid amount to Deposit Account No. 19-0741. If any extensions of time are needed for timely acceptance of papers submitted herewith, Applicant hereby petitions for such extension under 37 C.F.R. §1.136 and authorizes payment of any such extensions fees to Deposit Account No. 19-0741.

Respectfully submitted,

Date 6-30-08

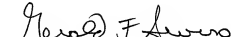
By 

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EXHIBIT I

Gene Therapy (1999) 6, 1835–1844

http://www.stockton-press.co.uk/gt

Taking lessons from dendritic cells: multiple xenogeneic ligands for leukocyte integrins have the potential to stimulate anti-tumor immunity

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Expression of large numbers of different costimulatory integrin ligands (CILs) attributes dendritic cells with an ability to induce primary anti-tumor immune responses. Here, we show that optimized gene transfer of the xenogeneic (human) CILs VCAM-1, MAdCAM-1 and ICAM-1 causes rapid and complete rejection of established mouse EL-4 tumors, and generates prolonged systemic anti-tumor immunity, whereas human E-cadherin weakly slows tumor growth. In each case the immune response was mediated by CD8⁺ T cells and NK cells, accompanied by augmented tumor-specific cytolytic T cell (CTL) activity involving both the perforin and Fas-ligand pathways. Adoptive transfer of splenocytes from cured mice rapidly cleared established tumors in recipients. The mechanism for CIL-mediated immunity is unknown, but may involve CTL-facilitated tumor lysis, since CTLs were generally twice as efficient at killing CIL-transfected tumor cells than parental tumor cells. Optimized CIL-based gene therapy may provide an approach to complement or replace conventional DC adoptive cell therapy for suppressing tumor growth.

Keywords: *cancer gene therapy; cancer; xenogeneic costimulatory integrin ligands; cytolytic T cells; NK cells; systemic anti-tumor immunity*

Introduction

Dendritic cells can provide valuable lessons in how to fight cancer, as they initiate the primary immune response and control the functions of effector B and T cells.[1] The efficacy of DCs in T cell binding and activation relates, in part, to their expression of high levels of a large number of different cell adhesion molecules (CAMs)[2–4] capable of costimulating T cell proliferation, including CD58/LFA-3, VCAM-1, ICAM-1, ICAM-3,[5] MAdCAM-1,[6] B7.1, B7.2, 4–1BB ligand,[7] RANK ligand,[8] MRC-OX-2,[9] and E-cadherin.[10] Such DC-derived immunoregulatory molecules could be employed to combat cancer and inflammatory disease, and could offer improved immunotherapeutic benefit over and above that already demonstrated with living DC-based vaccines.[11]

Transfection of the CD28/CTLA-4 ligands B7.1 and B7.2 into immunogenic tumor cells attributes such cells with an ability to present their tumor antigens and to generate anti-tumor cytotoxic T lymphocytes (CTL), whereas the immune system remains completely ignorant of the parental tumor cells.[12–15] Similarly, transduction of tumor cells with the costimulatory integrin ligand (CIL) ICAM-1 (ligand for LFA-1 integrin), and the tumor necrosis factor-like ligand 4–1BBL suppresses tumorigenicity and generates protective anti-tumor immunity.[16,17] Thus, gene transfer into tumors of three DC costimulatory molecules, which have been reported to stimulate different costimulatory pathways, leads to the generation of anti-tumor immunity.

We have demonstrated that recombinant solubilised immunoglobulin Fc-fusion forms of mouse MAdCAM-1, human VCAM-1 (ligands for VLA-4 and LPAM-1 integrins), and mouse E-cadherin (ligand for HML-1 integrin), in addition to human ICAM-1, mouse B7.1, and human B7.2 can costimulate the activation of CD4⁺ T cells *in vitro*.[18,19] VCAM-1, and ICAM-1 were found to be the most potent costimulators, and interestingly VCAM-1, MAdCAM-1, and ICAM-1 have the unique ability to costimulate 'remotely', that is they retain their ability to costimulate when presented on an entirely different surface from the stimulating anti-CD3.[18] We have therefore analysed the effects of VCAM-1, MAdCAM-1 and E-cadherin expression on tumor growth *in vivo*, revealing that multiple CIL have anti-tumor activity, and could be employed to either enhance or replace conventional DC adoptive therapy.

Results

Optimization of gene transfer of CILs to induce rejection of established tumors

EL-4 tumors were established in mice and injected with B7.1 and ICAM-1 DNA/liposome complexes diluted in water, PBS, 5% glucose, or 5% glucose in 0.01% Triton X-100 to determine the optimal diluent for gene transfer. The latter diluent markedly enhanced the anti-tumor activity of both B7.1 and ICAM-1, such that tumors began shrinking within 2 days. The rate of tumor rejection was significant 7 ($P < 0.05$) and highly significant 14 ($P <$

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0.001) days after gene injection, and by day 21 all tumors had virtually disappeared (Figure 1a). In contrast, all other diluents led to slow, but progressive tumor growth. Gene transfer of 60 μg plasmid DNA appeared optimal (Figure 1b), and was used with the 5% glucose/0.01% Triton X-100 throughout the following study.

Multiple CILs stimulate anti-tumor immunity and generate a systemic memory response

Human VCAM-1, ICAM-1, and human and mouse MAdCAM-1 and E-cadherin were examined for their ability to stimulate anti-tumor immune responses, and were compared with the established anti-tumor agents mouse B7.1 and human B7.2. Established tumors (0.1–0.2 cm) treated with B7.1, B7.2, VCAM-1, human MAdCAM-1, and ICAM-1 failed to grow ($P < 0.001$) (Figure 2a), and mice remained tumor-free for at least 2 months. In contrast, mouse MAdCAM-1 significantly ($P < 0.05$) slowed tumor growth, whereas growth inhibition by human E-cadherin was not significant ($P > 0.05$). Tumors treated with mouse E-cadherin and empty vector grew unchecked. Gene transfer resulted in the expression of CILs in >80% of cells (Figure 2b; similar results were also obtained with the FITC-labelled anti-mouse B7.1 mAb 1G10, data not shown).

Animals previously cured by gene therapy completely rejected the challenge of 1×10^5 parental tumor cells for the 36 days they were monitored (Table 1). In contrast, uncured animals whose tumors had been treated previously with human E-cadherin, failed to reject the parental tumor challenge (data not shown). As expected all animals failed to resist the challenge of the very large tumor burden of 5×10^7 cells. However human MAdCAM-1, ICAM-1, VCAM-1, B7.2 and mouse B7.1 did provide some protection (Table 1) since all mice survived to 36 days with small tumors, whereas untreated control mice subjected to the primary tumor challenge (Figure 2a) had to be killed as tumors reached 1 cm within 18–20 days.

Gene transfer of CILs stimulates tumor-specific cytolytic T cell activity, which can be adoptively transferred to cure recipient animals

The anti-tumor CTL activity of splenocytes obtained 28 days following gene transfer was significantly ($P < 0.001$) augmented in animals treated with ICAM-1, VCAM-1, human MAdCAM-1, and B7.1 and B7.2, versus those receiving empty vector or vehicle alone (Figure 3). Mouse MAdCAM-1 and human E-cadherin generated less CTL activity, whereas mouse E-cadherin had negligible effect. Anti-tumor CTL activity was also measured 36 days following challenge with parental tumor cells (Table 1). Challenge with 1×10^5 tumor cells generated profiles very similar to those measured 28 days after initial gene transfer. Surprisingly, challenge with the large tumor burden of 5×10^7 cells significantly ($P < 0.05$) suppressed the generation of anti-tumor CTL activity by as much as 46 to 80% compared with CTL activity generated with the small tumor burden (1×10^5 cells).

Adoptive transfer of 2×10^8 splenocytes from VCAM-1, ICAM-1, B7.1 and B7.2 treated mice into recipients bearing established EL-4 tumors resulted in the rapid and complete regression of all tumors (Figure 4). Splenocytes from human MAdCAM-1-treated mice caused a significant ($P < 0.001$) delay in tumor growth, whereas those obtained from mouse MAdCAM-1, and mouse and

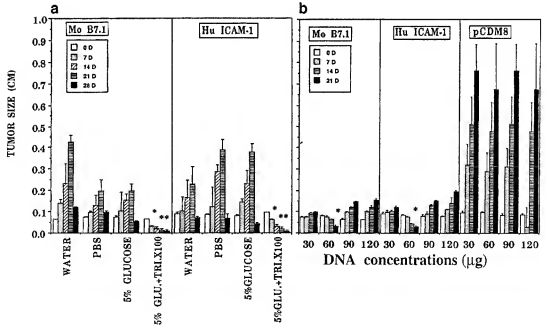

*Figure 1 Tumor rejection depends on the DNA diluent, and the gene dosage used for in situ gene transfer. Established tumors approximately 0.1 cm in diameter, were injected with DOTAP liposomes containing costimulator DNA resuspended in an array of different diluents, including PBS; 5% glucose; and 5% glucose in 0.01% Triton X-100 (a). Different amounts (30–120 μg) of DNA were included in the delivery vehicle (b). The empty expression vector pCDM8 served as a control. The sizes (cm; mean of two perpendicular diameters) of tumors was monitored for 21 days following gene transfer. *Indicates a significant difference at $P < 0.05$ from other groups, whereas ** indicates a highly significant difference at $P < 0.001$.*

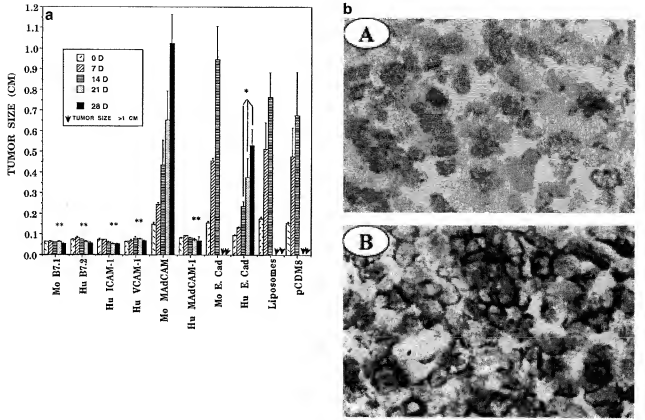

*Figure 2 Multiple CILs cause rapid rejection of established tumors. (a) Established tumors approximately 0.1 cm in diameter were injected with DOTAP liposomes containing 60 μg of each costimulator DNA. Control animals received empty pCDM8 vector, or liposomes alone. The sizes (cm) of tumors were monitored for 28 days following gene transfer. Mice were killed if tumors reached more than 1 cm in diameter (denoted by vertical arrows). The experiment was repeated twice (n = 6). *Indicates a significant difference at $P < 0.05$ from other groups, whereas ** indicates a highly significant difference at $P < 0.001$. (b) Immunohistochemical detection of B7.1 expression in a biopsy of tumor injected with (A) vector alone and (B) B7.1 cDNA expression vector. Illustrated is a representative of a tissue section (magnification ×100) stained with the anti-mouse B7.1 mAb 1G10. The reaction was developed with Sigma FAST with $CoCl_2$ enhancer tablets, generating a blue/blue-black stain. The section was prepared 2 days after B7.1 gene transfer.*

Table 1 Assessment of the anti-tumor memory response: large tumor burdens suppress the CTL response

DNA injected (60 μg)	*No. EL-4 cells*	*Animals with tumor after 36 days*			*Detectable tumor*	*CTL activity[a] E:T ratios[b]*			
		<0.1	*>0.1–0.3*	*>0.3*		*100:1*	*50:1*	*25:1*	*12:1*
B7.1	1×10^5	—	—	—	0/6	61.1 ± 5.2	30.0 ± 4.7	20.0 ± 1.2	16.0 ± 1.3
	5×10^5	2/6	2/6	2/6	6/6	28.3 ± 2.3*	10.3 ± 2.3*	5.5 ± 0.6*	4.4 ± 1.0*
B7.2	1×10^5	—	—	—	0/6	58.1 ± 4.4	35.1 ± 2.4	20.1 ± 1.3	12.1 ± 1.2
	5×10^5	3/6	1/6	2/6	6/6	23.4 ± 1.2*	10.3 ± 1.0*	4.3 ± 0.9*	3.4 ± 1.0*
ICAM-1	1×10^5	—	—	—	0/6	55.1 ± 2.3	28.1 ± 1.3	16.2 ± 0.7	8.3 ± 0.4
	5×10^5	2/6	1/6	3/6	6/6	21.6 ± 1.5*	10.2 ± 1.4*	4.6 ± 1.0*	3.2 ± 0.5*
VCAM-1	1×10^5	—	—	—	0/6	62.3 ± 3.9	40.3 ± 2.3	26.2 ± 2.4	10.2 ± 1.2
	5×10^5	2/6	1/6	3/6	6/6	22.2 ± 1.0*	10.3 ± 1.2*	6.4 ± 0.5*	4.4 ± 1.9*
Hu MAd CAM-1	1×10^5	—	—	—	0/6	51.3 ± 2.7	26.9 ± 1.6	15.3 ± 1.2	14.3 ± 1.4
	5×10^5	1/6	2/6	3/6	6/6	18.3 ± 1.6*	9.3 ± 1.0*	5.4 ± 0.9*	4.3 ± 0.8*

Mice that were cured of their tumors using the indicated costimulatory genes were rechallenged with either 1×10^5 or 5×10^5 parental tumor cells. Tumor occurrence, size of the tumor and CTL activity of splenocytes were measured after 36 days.
*Indicates a significant difference at $P \leq 0.05$ from mice challenged with 1×10^5 EL4 cells.
[a]Values were presented as mean ± s.d. of triplicate wells from at least two experiments having six mice in each group.
[b]Effector:target cell ratio.

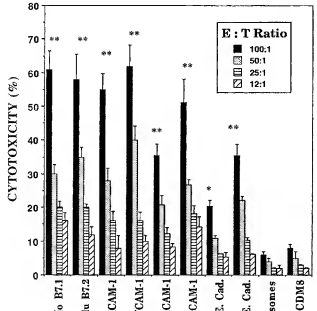

*Figure 3 Comparison of anti-tumor CTL activity generated by gene transfer of different costimulators. Splenocytes were removed from animals 28 days following CIL gene transfer and were tested for cytolytic activity against EL-4 tumor cells. The percentage cytotoxicity is plotted against various effector to target (E:T) ratios. Control animals received empty pCDM8 vector or liposomes alone. * and ** indicate a significant and highly significant difference from control groups of mice at $P < 0.05$ and $P < 0.001$, respectively.*

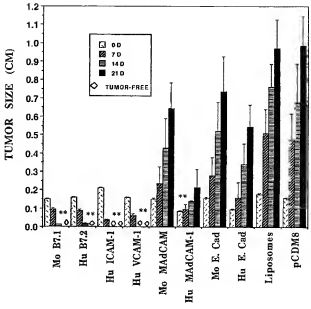

*Figure 4 Eradication of established tumors by adoptive transfer of anti-tumor CTL from treated mice. Splenocytes (2×10^8) were adoptively transferred by intratumoral and i.p. injection, from costimulatory CAM-treated and control mice to recipient mice bearing established tumors (0.1–0.2 cm diameter). The sizes (cm) of tumors were monitored for 21 days following adoptive transfer. ** Indicates a highly significant difference at $P < 0.001$ from control groups of mice.*

human E-cadherin-treated mice afforded no significant ($P > 0.05$) protection.

Composition of the effector cell population responsible for CIL-mediated immunity

The primary rejection of human VCAM-1, ICAM-1, MAdCAM-1, B7.2 and mouse B7.1-treated tumors ($P < 0.001$), and inhibition of tumor growth by human E-cadherin and mouse MAdCAM-1 was mediated by $CD8^+$ T cells (Figure 5b), NK cells (Figure 5c), and only partly by $CD4^+$ T cells (Figure 5a). Simultaneous depletion of both $CD8^+$ T cells and NK cells resulted in completely unchecked tumor growth, similar to that in untreated controls (Figure 5d). Tumors began to regress 1 week after suspending anti-CD8, and anti-NK cell antibody treatment, but the mice never became tumor-free during the 21 day period they were monitored (data not shown).

CILs facilitate initial tumor cell lysis

To assess whether expression of CILs facilitates CTL-mediated tumor cell lysis, an *in vitro* CTL killing assay was devised where splenocytes from animals with B7.1-treated tumors were mixed with disaggregated EL-4 cells that had been isolated (2 days after gene transfer) from the tumors of animals treated with a variety of CILs. Disaggregated EL-4 cells expressed the CAMs at high levels where 80–85% of cells were positive (Figure 6a). At an effector to target ratio of 25:1, CTL showed highly significant ($P < 0.001$) killing of tumor cells transfected with human VCAM-1, ICAM-1, MAdCAM-1, B7.2 and mouse B7.1 than parental EL-4 cells (Figure 6b). In general, they were twice as efficient at killing transfected tumor cells *versus* parental cells. Mouse E-cadherin and MAdCAM-1 expressing tumor cells were also significantly ($P < 0.05$) better targets. Abrogation by anti-CIL antibodies of the enhanced sensitivity of transduced tumor cells to CTL-mediated lysis was highly significant ($P < 0.001$) (Figure 6b).

Anti-tumor CTL employ both the Fas-ligand and perforin pathways to kill EL-4 target cells

The *in vitro* CTL killing assay was utilized to evaluate the cytotoxic mechanisms of CIL-mediated tumor rejection. Inclusion of EGTA/$MgCl_2$, which specifically blocks the Ca^{++}-dependent perforin pathway, led to partial inhibition of tumor-specific CTL activity (Figure 7). Pretreatment of splenocytes with the neutralizing anti-Fas-ligand mAb MFL3 was even more effective at blocking CTL activity, whereas simultaneous blocking of both the perforin and Fas-ligand pathways dramatically inhibited ($P < 0.001$) the CTL activity of all animals examined.

Discussion

Multiple CILs have therapeutic potential in treating cancer

Here we have demonstrated for the first time that gene transfer of human VCAM-1 and MAdCAM-1 into established tumors is as effective as B7.1, B7.2 and ICAM-1, which have previously been demonstrated to have anti-tumor activity.[12–16] Successful treatment depended on the amount of DNA for gene transfer, the DNA diluent, the

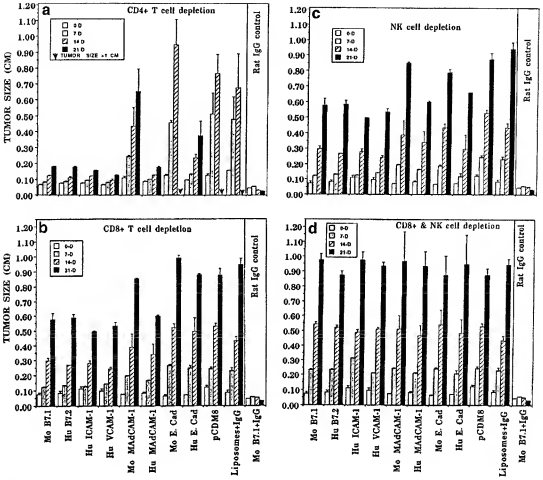

Figure 5 Anti-tumor immunity is largely mediated by CD8⁺ T cells and NK cells. Mice were treated with the anti-CD4 mAb GK1.5 (a), the anti-CD8 mAb 53–6.72 (b), and the anti-NK cell mAb PK136 (c) 4 days before gene transfer, and every alternate day for the duration of the experiment. The anti-CD8 and anti-NK cell antibodies were also administered simultaneously (d). The sizes (cm) of tumors were monitored for 21 days following gene transfer. As can be seen, the anti-CD8 and anti-NK mAbs almost completely abrogated tumor rejection, whereas the anti-CD4 mAb had little effect on tumor rejection.

xenogenicity of CILs, and tumor size. Whereas a range of doses of each costimulator gene were inhibitory for tumor growth, 60 μg of DNA injected into 1 mm tumors was optimal for rapid tumor regression, reflecting a requirement to maximise transfection efficiencies or optimise expression of CILs. The diluent 5% glucose in 0.01% Triton X-100 was optimal, as described previously to introduce a reporter gene into mouse melanoma BL6 tumors.[20]

Xenogeneic forms of CILs were found to have greater anti-tumor activity than syngeneic forms. Thus whereas mouse MAdCAM-1 only weakly slowed tumor growth, human MAdCAM-1 caused rapid tumor rejection, and generated anti-tumor systemic immunity. Similarly, mouse E-cadherin was totally without effect, but its human homologue very weakly slowed tumor growth. These results are in agreement with those of Wei *et al*[16] who found that rat tumor cell lines engineered to express human ICAM-1 stimulated anti-tumor immunity in rats, whereas rat ICAM-1-expressing tumor cells were not effective. It was argued that human ICAM-1 provides sufficient stimulatory signals due to both its costimulatory function, and its inherent antigenicity as a foreign antigen. In contrast in a mouse model of neuroblastoma, transfection of the mouse ICAM-1 gene into tumors reduced their tumorigenicity.[21]

Further experiments are required to dissect the contributions of 'foreigness' *versus* T cell costimulation in generating anti-tumor immunity. Mouse B7.1 generates protection against mouse tumors, suggesting that its T cell costimulatory function can be sufficient. In contrast human E-cadherin is only very weakly protective, indicating that 'foreigness' alone is not sufficient. Xenogeneic CILs may somehow break tolerance and thereby overcome immunological ignorance of tumors. Immunization of mice with purified syngeneic melanoma antigen gp75 failed to elicit antibody or cytotoxic T cell responses, whereas immunization with human gp75 elicited autoantibodies to syngeneic gp75 leading to rejection of metastatic melanomas.[22] In our experiments, anti-tumor humoral immunity was not detected, as serum taken from cured animals had no protective effect when injected into recipient animals (data not shown).

We have consistently found that gene transfer of CILs

into tumors less than 0.3 cm in diameter causes rapid tumor rejection, whereas tumors become increasingly refractory to treatment as their size increases. Larger tumors appear actively to suppress or avoid the immune response, since challenge of cured animals with large tumor burdens led to reductions in the levels of anti-tumor CTL activity compared with the response to smaller burdens. The mechanisms responsible are unknown, but could include the increased secretion of immunosuppressive cytokines such as TGF-β,[23] or metalloproteases which release latent TGF-β from extracellular stores, loss of tumor antigens, or acquisition of resistance to killing. Alternatively, we have observed that EL-4 cells begin to express cell-surface Fas-ligand as cell density increases, whereas Fas is expressed at a constant level (data not shown). Fas-expressing EL-4 tumor cells may kill responding anti-tumor CTL,[24,25] and thereby impair the anti-tumor immune response. A further explanation would be that certain EL-4 cells within the larger secondary tumor challenges may have distinct antigenic profiles unrelated to that of cells in the original primary tumor challenge, and hence are not susceptible to killing by anti-tumor memory CTL. Repeated CIL gene transfer into tumor regrowths arising from antigenic drift could potentially exhaust the heterogeneity of a tumor, and provide protective immunity for the life of an animal. CIL gene transfer into tumors arising from rechallenge of cured animals with a large tumor burden led once again to complete tumor rejection (data not shown), in support of the latter notion.

That CILs facilitate tumor cell lysis was shown by the fact that EL-4 cells transduced with the various CILs were better targets for anti-tumor CTL than unmodified EL-4 cells. There is now a large body of evidence showing that ICAM-1 expression on tumor cells enhances tumor susceptibility to lysis by CTL,[21,26–28] and more recently ICAM-1 and B7.1 expression was found to induce MHC-unrestricted killing of target cells by promiscuous CTLs.[29] Low expression of VCAM-1 on the lymphokine-activated killer (LAK)-resistant cell line OKM-2T has been suggested to be partly involved in resistance to lysis by LAK cells.[30] Our results demonstrate for the first time that MAdCAM-1 and E-cadherin also play a role in target cell lysis by CTL.

While all the CAMs seem to increase the cytotoxicity of CTL towards the tumor cells, not all are effective in the rejection of the tumor. They presumably increase cytotoxicity by enhancing conjugate formation between tumor cells and antitumor CTL. However, conjugate formation alone may not be sufficient initially to generate anti-tumor CTL. We have previously demonstrated that E-cadherin[19] and MAdCAM-1[18] are able to costimulate T cell proliferation, but again this may not be sufficient as E-cadherin and MAdCAM-1 are poor stimulators of anti-tumor activity against EL-4 cells. Other key recognition or signalling events mediated by the CAMs must clearly be involved to stimulate efficient anti-tumor activity. CAMs, like ICAM-1 and VCAM-1 that cause tumor rejection, induce the upregulation of CD28 and CTLA-4 on T cells,[31] and potentially other signalling molecules such as cytokines like GM-CSF[32] that may enhance T cell costimulation and tumor rejection.

The mechanism of CIL-mediated anti-tumor immunity

Adoptive transfer of splenocytes from cured mice into recipient animals confirmed that CIL anti-tumor activity was mediated by cellular immunity. Antibody depletion of leukocyte subsets revealed that the anti-tumor immune response was largely mediated by $CD8^+$ T cells and NK cells, as has been noted previously for B7.1 and ICAM-1-mediated anti-tumor immunity.[33] Both NK and $CD8^+$ T cells may be necessary to kill EL-4 cells that express different levels of MHC class I, where NK cells would be expected to kill EL-4 cells expressing little or no MHC class I. The EL-4 cells used in the present study contained mixed populations either expressing or lacking MHC class I (Figure 8). There was a minor contribution from $CD4^+$ T cells, in accord with the fact that some EL-4 cells expressed MHC class II molecules (Figure 8). Of note was the fact that the nature of the anti-tumor immune response appeared identical regardless of the identity of the costimulatory molecule used for gene transfer. It is possible that B7.1, B7.2, VCAM-1, ICAM-1 or MAdCAM-1 function by initially breaking tolerance or overcoming immunological ignorance of the tumor leading to tumor cell lysis, but from thereafter the CAMs become

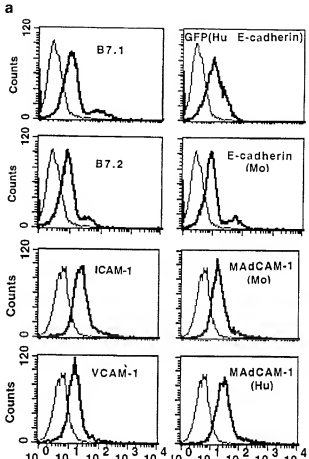

Figure 6 Costimulatory CAMs play a role in facilitating cell lysis by anti-tumor CTL. (a) CAM expression on EL-4 cells recovered from treated tumors. EL-4 cells isolated from the tumors of B7.1, B7.2, ICAM-1, MAdCAM-1, and E-cadherin-treated mice 2 days following gene transfer were stained with mAbs specific for the individual CAMs (thick lines), and with an isotype-matched control mAb (thin line). Cotransfection with GFP was used to estimate efficiency of transfection with the hu-E-cadherin expression vector.

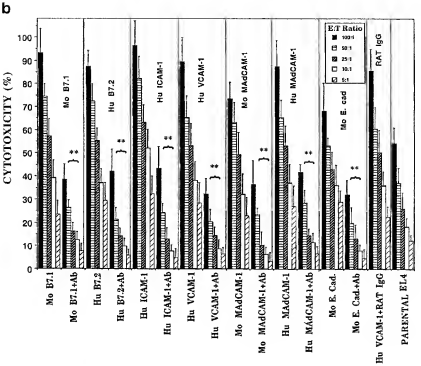

*Figure 6 Continued. (b) Shown is a representative experiment in which parental EL-4 cells and cells isolated from the tumors of B7.1, B7.2, ICAM-1, MAdCAM-1, and E-cadherin-treated mice 2 days following gene transfer were mixed in different effector to target (E:T) ratios with splenocytes from B7.1-treated mice. Cytotoxicity assays were also performed in the presence of saturating concentrations of antibodies to each costimulatory CAM, or rat IgG control antibody (latter control shown only for VCAM-1, but similar results obtained for other CAMs). Similar cytotoxicity profiles were obtained with splenocytes isolated from mice treated with the other costimulatory CAMs. ** Indicates a highly significant difference at $P < 0.001$ from control groups of mice.*

superfluous as tumor antigens are presented by professional APCs resulting in a common mechanism of tumor destruction. However, a detailed analysis of the tumor infiltrate will be required to determine whether the different CAMs elicit different immune mechanisms. A previous study had noticed that mouse B7.1 was superior to mouse B7.2 in the induction of anti-tumor immunity in a mouse model of leukemia, but this was not the case in our tumor model where the human homologue of B7.2 was employed.[34]

Anti-tumor CTL activity stimulated by each of the costimulatory CAMs involved both the perforin and Fas-ligand pathways, where the Fas-ligand pathway predominated. Treatment of cells with a combination of EGTA and anti-Fas-ligand mAb to block both pathways, almost completely inhibited the activity of anti-tumor CTLs. Residual cytolytic activity that remained may be due either to incomplete inhibition of the perforin and Fas-ligand pathways or to TNF-α-mediated cytotoxicity, as B7.1 and ICAM-1 have been shown to induce cytotoxic levels of TNF on T cells.[35]

In summary, these results demonstrate that multiple xenogeneic CILs have therapeutic potential in the treatment of immunogenic cancers. Optimized gene transfer of xenogeneic CILs is a very potent method for causing the rejection of small tumors, and could be used in combination with tumor-size reducing agents such as cell cycle inhibitors, anti-angiogenic agents, and chemotherapy for the treatment of advanced cancer. CILs could be used to supplement, enhance or supercede conventional DC adoptive immunotherapy of cancer.

Materials and methods

Mice and cell lines

Female C57BL/6 mice, 6–8 weeks old, were obtained from the Animal Resource Unit, School of Medicine and Health Science, University of Auckland, Auckland, New Zealand. The EL-4 thymic lymphoma, which is of C57BL/6 (H-2b) origin was purchased from the American Type Culture Collection (Rockville, MD, USA). It was cultured *in vitro* at 37°C in DMEM medium (Gibco BRL, Grand Island, NY, USA), supplemented with 10% foetal calf serum, 50 U/ml penicillin/streptomycin, 2 mM L-glutamine, 1 mM pyruvate. The cell line was heterogeneous with regard to MHC expression, where subpopulations either expressed or lacked MHC class I and II molecules (Figure 8).

Antibodies and cDNAs

Mouse hybridomas secreting mAbs against human VCAM-1 (VIII-6G10 mAb),[36] mouse CD8 (53-6.72 mAb), CD4 (Gk1.5 mAb), and NK cells (PK136 mAb) were purchased from the American Type Culture Collection. The rat hybridoma cell line MECA-367[37] which secretes a mAb against mouse MAdCAM-1 was kindly provided by Dr Eugene Butcher, Stanford University, Stanford, CA,

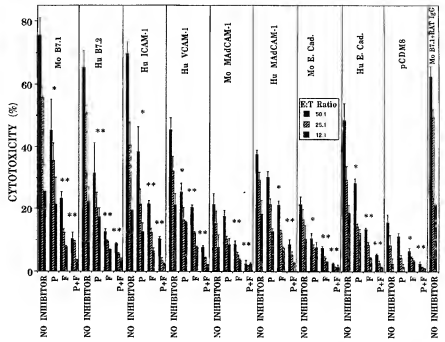

*Figure 7 Anti-tumor immunity is mediated by both the Fas ligand and perforin pathways. Cytotoxicity assays using different target to effector (E:T) ratios were performed as described in Figure 3, but in the presence of 5 mM EGTA/4 mM $MgCl_2$ to measure the involvement of the perforin pathway, and in the presence of the anti-Fas ligand mAb MFL3 to record Fas/Fas-ligand involvement. Rat IgG served as a control antibody. P denotes inhibition of the perforin pathway; F indicates inhibition of Fas ligand; and P + F represents simultaneous inhibition of both pathways. * and ** indicate a significant and highly significant difference from control groups of mice at $P < 0.05$ and $P < 0.001$, respectively.*

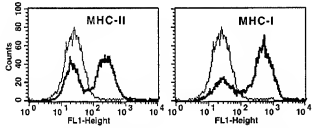

Figure 8 EL-4 cells express MHC class I and II. EL-4 cells were stained either with FITC-labelled TH81.A5 mAb (IgG2a; MHC class II), the H58A mAb (IgG2a; MHC class I), or with the FITC-labelled isotype matched control IgG2a mAb 2G5 to human HML-1 (lighter line), and MHC class I and II expression evaluated by flow cytometry.

USA. The polyclonal anti-mouse E-cadherin antiserum has been described elsewhere.[19] A rabbit polyclonal antibody against human MAdCAM-1 was raised to a MAdCAM-1-GST fusion protein encoding nucleotides 272 to 562 of the published sequence[38] (accession No. U82483). Antibodies against mouse B7.1 (1G10), human B7.2 (IT2.2), and human ICAM-1 (HA58) were purchased from Pharmingen, San Diego, CA, USA. Anti-mouse MHC Class I (H58A) and II (TH81A5) mAbs were purchased from Veterinary Medical Research Development, Pullman, WA, USA. The 2G5 mAb to human HML-1 was obtained from Immunotech, Marseille, France). Complementary DNA encoding full-length human VCAM-1 was purchased from R&D Systems, Abingdon, UK; B7.2[39] was provided by Dr G Freeman, Dana Farber Cancer Institute, Boston, MA, USA; human ICAM-1 was donated by Dr J Ni, Human Genome Sciences, Rockville, MD, USA; mouse B7.1[13] was provided by Dr P Linsley, Bristol-Myers-Squibb, Seattle, WA, USA; mouse MAdCAM-1[40] was generously donated by Dr Eugene Butcher, Stanford University; mouse E-cadherin[41] was provided by Dr M Takeichi, Kyoto University, Kyoto, Japan; whereas human E-cadherin[42] was kindly donated by Drs D Rimm and J Morrow, Yale University School of Medicine, New Haven, CT, USA. We have previously reported the cloning and characterization of human MAdCAM-1 cDNA.[38]

Gene transfer of CILs and measurement of anti-tumor systemic immunity

Plasmid expression vectors in pCDM8 were prepared by cesium chloride gradient centrifugation, and diluted to 600 μg/ml in a solution of 5% glucose in 0.01% Triton X-100, unless indicated otherwise. They were mixed in a ratio of 1:3 (wt:wt) with DOTAP cationic liposomes (Boehringer Mannheim, Mannheim, Germany). Tumors were established by injection of 1×10^5 EL-4 tumor cells into the left flank of mice, and growth determined by measuring two perpendicular diameters. Animals were killed when tumors reached more than 1 cm in diameter, in accord with Animal Ethics Approval (University of Auckland). Tumors reached 0.1 to 0.3 cm in diameter after approximately 14 days, and were injected at multiple sites with 100 μl of DNA (60 μg) per liposome complex. Cured mice were rechallenged 3 weeks after gene transfer by injecting either 1×10^5 or 5×10^7 EL-4 cells (0.1 ml) subcutaneously into the opposing flank (right flank). All experiments included six mice per treatment group and each experiment was repeated at least once. Statistical analysis was determined by the Student *t* test, where

$P < 0.05$ was considered significant, and $P < 0.001$ highly significant.

Immunohistochemistry

Tumor cryosections (10 μm) made 2 days following gene transfer were blocked with horse serum and stained with anti-CAM mAbs using the VECTASTAIN Universal Quick kit (Vector Laboratories, Burlingame, CA, USA). They were developed with Sigma FAST DAB (3, 3'-diaminobenzidine tetrahydrochloride) with $CoCl_2$ enhancer tablets (Sigma). Tissue sections were also treated with the 1G10 mAb, then FITC-conjugated goat anti-rat IgG (Sigma), and examined by fluorescence microscopy.

FACScan analysis of CAM gene expression

Tumors were excised 2 days following gene transfer, and a single cell suspension prepared by collagenase digestion. Tumor cells were cultured for a further 2 days and stained with anti-CAM mAbs, and FITC-conjugated goat anti-rat IgG (Sigma); or with the FITC-labelled isotype-matched control IgG2a mAb 2G5 to human HML-1, and evaluated by flow cytometry. Since we have no antibody reagent against human E-cadherin, transfection efficiency of the human E-cadherin expression vector was estimated by cotransfection with the pAAV/CMV-GFP vector. Green fluorescence for GFP expression was recorded in the FITC emission channel. Cells transfected with empty vector alone served as controls.

Adoptive transfer of CIL-stimulated CTL

Splenocytes isolated as described previously[43] were resuspended in Hank's balanced salt solution containing 1% FCS, and stimulated with 5 μg/ml PHA and 100 U/ml recombinant mouse IL-2 for 4 to 5 days. Animals bearing tumors, established for 14–20 days, received both intratumoral and intraperitoneal (i.p.) injections of 2×10^8 cultured splenocytes.

Cytotoxicity assays

Splenocytes were harvested 28 days following initial gene transfer, or 42 days after a parental tumor challenge, and incubated at 37°C with EL-4 target cells in graded E:T ratios in 96-well round-bottom plates. After a 4-h incubation, 50 μl of supernatant was collected, and lysis was measured using the Cyto Tox 96 Assay Kit (Promega, Madison, WI, USA). Background controls for non-specific target and effector cell lysis were included. After background subtraction, percentage of cell lysis was calculated using the formula: 100 × (experimental-spontaneous effector-target spontaneous target/maximum target-spontaneous target). The roles of the Fas ligand and perforin pathways in facilitating tumor cell lysis was tested by pre-incubating the effector CTL with 12 μg/ml Fas ligand neutralizing antibody MFL3 (Pharmingen, San Diego, CA, USA) at 37°C, and by adding 5 mM EGTA/4 mM $MgCl_2$ to mixes of the target and effector cells, respectively.

Analysis of the roles of CIL in tumor cell lysis

Tumors were excised 2 days following gene transfer, and injected with 110 U collagenase (1.1 ml), and tumor cells isolated by homogenization, further collagenase treatment, and centrifugation. Cells were cultured for 6 days, and the levels of expression of CILs and B7 molecules assessed by FACScan analysis. They were then used as CTL targets as described above.

Depletion of leukocyte subsets

Mice were depleted of $CD8^+$, and $CD4^+$ T cells and NK cells by i.p. and intravenous injection 4 days before gene transfer, and thereafter every alternate day with 300 μg (0.1 ml) of the 53–6.72 (anti-CD8), Gk1.5 (anti-CD4), and PK136 (anti-NK) mAbs. Rat IgG (Sigma) was used as a control antibody. Antibodies were an ammonium sulphate fraction of ascites, which titered to at least 1:2000 by FACS (Becton Dickinson, San Jose, CA, USA) staining splenocytes. Depletion of individual leukocyte subsets was found to be more than 90% effective, as determined by FACScan analysis.

Statistical analysis

Results were expressed as mean values ± standard deviation (s.d.), and a Student's t test was used for evaluating statistical significance. A value less than 0.05 ($P < 0.05$) was used for statistical significance.

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